EXHIBIT B

viruses as expression vectors
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poxviruses are widely used for the cytoplasmic expression of recombinant genes in mammalian cells. Recent improvements allow high expression and simplify the integration of multiple foreign genes. Vaccinia virus mutants and other poxviruses that undergo abortive infection in mammalian cells are receiving special attention because of their diminished cytopathic effects and increased safety. Now replicating and 'non-replicating' vectors, encoding the bacteriophage T7 RNA polymerase for transcription of recombinant genes, have been engineered.

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Abbreviations
β-gal	β-galactosidase
gpt	xanthine-guanine phosphoribosyltransferase
gus	β-glucuronidase A gene
MVA	modified vaccinia virus Ankara
rVV	recombinant VV
VV	vaccinia virus

Introduction
The purpose of this review is to highlight recent progress r the construction and use of poxvirus vectors; earlier studies are described and referenced elsewhere [1•–3•]. Recombinant gene expression by vaccinia virus (VV), the prototypic member of the orthopoxvirus genus of the Poxviridae family, was first demonstrated in 1982 [4,5]. Special features of the VV vector system include a capacity to stably integrate over 25,000 base pairs of foreign DNA into the viral genome without loss of infectivity, a cytoplasmic site of gene expression, and a wide vertebrate host range. Other poxviruses, including members of the avipoxvirus genus, however, have greater host specificity. The versatility, for both transient and stable expression, was extended by the incorporation of bacteriophage transcriptional systems into the vaccinia virus genome. Poxvirus expression systems have been used for analysis of protein structure/function relationships, protein processing and intracellular trafficking, antigen presentation, the determinants of cellular and humoral immunity, and as live recombinant vaccines.

Construction of recombinant vaccinia virus by homologous recombination
The original and still most widely used method of producing recombinant VV (rVV) is by homologous recombination. Cells are transfected with a transfer plasmid, which contains the recombinant gene under control of a VV promoter flanked by several hundred base pairs of VV derived DNA, and infected with VV. Recombination occurs between homologous sequences in the plasmid and viral genome. A variety of methods are available for the isolation of rVV including selection based on bromo deoxyuridine or antibiotic-resistance, detection of a reporter gene expressing a colour marker, complementation of a host range or small plaque phenotype, and direct antibody staining of plaques or DNA hybridization [6]. The E. coli β-glucuronidase A gene (gus), provides an alternative to β-galactosidase (β-gal) as a colour marker [7,8]. gus is a relatively small gene and fortuitously lacks commonly used restriction sites, thereby simplifying vector constructions. Furthermore, as the substrates for gus and β-gal are not cross-reactive, both reporter genes can be used for making rVV with multiple inserts. A gus–neo cassette provides both colour detection and antibiotic-resistance (T Shors, B Moss, unpublished data). A convenient E. coli xanthine-guanine phosphoribosyltransferase (gpt)–gus fusion protein has been described for similar use [9]. The reporter gene encoding green fluorescent protein has been incorporated into VV [10]. A particularly efficient selection method, based on the repair of the non-plaque-forming phenotype of a mutant VV, neither interrupts a vaccinia virus gene nor inserts a reporter or antibiotic selection marker [11].

The stable integration of a selectable marker precludes its use for selection of a second gene; in addition, extra genetic material may not be desirable in a rVV that is to be used for clinical trials. Schemes in which antibiotic-resistance or colour marker genes are integrated and then spontaneously deleted by recombination have been developed [12–14]. A modified procedure uses both gpt and β-gal for transient selection (F Scheiflinger, F Dorner, FG Falkner, personal communication). An alternative protocol, in which a separate non-integrating plasmid containing gpt is cotransfected with the transfer plasmid provides a several-fold enrichment of rVV by antibiotic selection [15].

Construction of rVV by In vitro ligation
The in vitro ligation of a foreign gene into the VV genome provides an alternative to homologous recombination [1•]. Since VV DNA is not infectious, the cells are transfected with rVV DNA and infected with a host-restricted helper

vipoxvirus or conditionally lethal VV. These techniques allow the efficient insertion of very large DNA fragments or even libraries of DNA fragments directly into the vaccinia virus genome. rVV genomes have been engineered with promoters and unique restriction endonuclease sites to facilitate cloning and expression [16,17].

Poxvirus promoters

Viral promoters are required for expression of foreign genes because poxviruses replicate in the cytoplasm and have their own transcriptional system. The promoters are about 30 base pairs long and come in three varieties (early, intermediate and late) and various strengths. Early promoters are expressed before viral DNA replication, whereas intermediate and late promoters are expressed successively after DNA replication [18•]. Despite their lower intrinsic activity, early promoters may be preferred in order to obtain expression before virus-induced cytotoxic effects and for cells that are not fully permissive. To achieve both early and high expression, many transfer vectors contain natural, modified or strong synthetic tandem early/late promoters [19•,20].

Replication-deficient vaccinia virus expression systems

VV is infectious and therefore use of a containment facility with a microbial safety cabinet and vaccination of laboratory workers may be recommended or required by some institutions. Two highly attenuated strains of vaccinia virus, NYVAC and modified vaccinia virus Ankara (MVA), have been approved by the National Institutes of Health (USA) intramural biosafety committee for use without a safety cabinet or vaccination. NYVAC was constructed by deleting 18 genes, some of which are involved in host-range and pathogenesis [2•]. In human cell lines, replication of NYVAC is blocked at an early stage, whereas productive infection is observed in African green monkey kidney (VERO) cells and chick embryo fibroblasts. MVA, by contrast, is blocked in virion assembly; consequently, late expression of recombinant proteins occurs even in non-permissive cells [1•]. MVA grows to high titers in chick embryo fibroblast and, surprisingly, in baby hamster kidney 21 cells but replicates only slightly or not at all in human and other mammalian cell lines tested because of multiple gene defects (MW Carroll, B Moss, abstract W41-4, 16th annual meeting of the American Society for Virology, Bozeman, MO, July 1997).

Recently, a mutated VV with a deleted uracil DNA glycosylase gene was constructed using a complementing cell line [21••]. The enzyme is required for DNA replication, so that prolonged early gene expression occurs in non-permissive cells. This defective virus may be useful as an expression vector. Controlled cross-linking of VV DNA, with psoralen and UV light, severely reduces viral infectivity while allowing expression of a recombinant gene regulated by a viral early promoter [22]. Under appropriate conditions, virus-induced cytotoxicity and

inhibition of host protein synthesis were reduced while recombinant gene expression was prolonged. Restrictions of the latter system include poor expression of genes with large UV target sizes and inability to employ late promoters for high expression.

Bacteriophage/vaccinia virus hybrid expression systems

The hybrid system is based on the integration of a bacteriophage RNA polymerase gene into the genome of VV for the specific expression of recombinant genes. Transient and stable forms of the expression system are available [1•]. In the widely used transient system, cells are infected with a rVV that expresses the bacteriophage T7 or bacteriophage SP6 RNA polymerase and are transfected with a plasmid that contains a gene under the control of a T7 or SP6 promoter. High expression is achieved when the encephalomyocarditis virus cap-independent ribosome binding site is present to enhance translation. In the stable form of the system, the T7 promoter-regulated gene is also incorporated into the VV genome. Recent modifications of this system provide stringent chemical or thermal inducibility of recombinant gene expression [23]. Another modification incorporates the cowpox virus host range gene into rVV, allowing efficient gene expression in normally nonpermissive Chinese hamster ovary cells [24]. The T7 RNA polymerase gene has been incorporated into the MVA strain of VV, allowing its use for transient gene expression in a defective vector system [25,26].

Other poxviruses as vectors

Poxvirus promoters and transcription factors are conserved among even distantly related poxviruses, so that the basic strategies developed for VV expression vectors can be employed for all members of the family. Host-specificity has been the major inducement leading to the development of alternative poxvirus vectors. The orthopoxviruses, raccoonpox [27] and ectromelia (mousepox) [28] may be useful vectors for wild-life vaccines or for basic studies of pathogenesis and immunity in the laboratory mouse, respectively. Members of the capripoxvirus and leporipox genera have been developed as vectors for cattle [29] and rabbits [30], respectively. Avipoxviruses, initially developed as host-range restricted vaccines for birds, have also been promoted as human vaccine candidates because of their safety and lack of immunological cross-reactivity with vaccinia virus [2•]. The stage at which fowlpox virus replication is blocked may vary for different non-avian cell lines, and both early and some late gene expression have been detected [31]. A recombinant fowlpox virus expressing the T7 RNA polymerase gene offers an alternative to rVV for the transient expression of recombinant genes [32].

Basic laboratory applications of poxvirus vectors

Investigators continually find novel and important uses for rVV vectors, so only a few recent examples are

al review of replicating and non-replicating poxvirus vectors that provide numerous references omitted from the present summary. Includes background information on the biological properties of poxviruses as ed to their use as vectors.

Paoletti E: Applications of pox virus vectors to vaccination: an update. Proc Natl Acad Sci USA 1996, 93:11349-11353.
recent review primarily of non-replicating poxvirus vectors developed as vaccine candidates by the author and collaborators.

3. Flexner C, Moss B: Vaccinia virus as a live vector for expression of immunogens. In New Generation Vaccines, 2nd edn. Edited by Levine MM, Woodrow GC, Kaper JB, Cobon GS. New York: Marcel Dekker; 1997:297-314.
An extensive review of recombinant vaccinia virus vectors, listing many that have been used for expression of immunogens in animal models.

4. Mackett M, Smith GL, Moss B: A selectable eukaryotic cloning and expression vector. Proc Natl Acad Sci USA 1982, 79:7415-7419.

5. Panicali D, Paoletti E: Construction of poxviruses as cloning vectors: insertion of the TK gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc Natl Acad Sci USA 1982, 79:4927-4931.

6. Earl PL, Moss B: Generation of recombinant vaccinia viruses. In Current Protocols in Molecular Biology. Edited by Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. New York: Greene Publishing Associates & Wiley Interscience; 1991:16.17.1-16.17.16.

7. Carroll MW, Moss B: E. coli β-glucuronidase (GUS) as a marker for recombinant vaccinia viruses. Bio-Techniques 1995, 19:352-355.

8. Howley PM, Spehner D, Drillien R: A vaccinia virus transfer vector using a GUS reporter gene inserted into the I4L locus. Gene 1996, 172:233-237.

9. Cao J, Upton C: gpt-gus fusion gene for selection and marker in recombinant poxviruses. Bio-Techniques 1997, 22:276-278.

10. Wu G-Y, Zou D-J, Cline HT: Infection of frog neurons with vaccinia virus permits in vivo expression of foreign proteins. Neuron 1995, 14:681-684.

11. Blasco R, Moss B: Selection of recombinant vaccinia viruses on the basis of plaque formation. Gene 1995, 158:157-162.

12. Falkner FG, Moss B: Transient dominant selection of recombinant vaccinia viruses. J Virol 1990, 64:3108-3111.

13. Isaacs SN, Kotwal GJ, Moss B: Reverse guanine phosphoribosyltransferase selection of recombinant vaccinia viruses. Virology 1990, 178:626-630.

14. Spehner D, Drillien R, Lecocq J-P: Construction of fowlpox virus vectors with intergenic insertions: expression of the β-galactosidase gene and measles virus fusion gene. J Virol 1990, 64:527-533.

15. Kurilla MG: Transient selection during vaccinia virus recombination with insertion vectors without selectable markers. Bio-Techniques 1997, 22:906-910.

16. Pfleiderer M, Falkner FG, Dorner F: A novel vaccinia virus expression system allowing construction of recombinants without the need for selection markers, plasmids and bacterial hosts. J Gen Virol 1995, 76:2957-2962.

17. Merchlinsky M, Eckert D, Smith E, Zauderer M: Construction and characterisation of vaccinia direct ligation vectors. Virology 1997, in press.

18. Moss B: Poxviridae: the viruses and their replication. In Fields Virology. Edited by Fields BN, Knipe DM, Howley PM. Philadelphia: Lippincott-Raven Publishers; 1996:2637-2671.
This introduction to the biology of poxviruses is recommended to anyone using poxvirus vectors.

19. Wyatt LS, Shors ST, Murphy BR, Moss B: Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model. Vaccine 1996, 14:1451-1458.
Comparison of strengths of several early/late promoters. Demonstrates immunogenicity of recombinant modified vaccinia virus Ankara by intranasal and intramuscular routes.

20. Chakrabarti C, Sisler JR, Moss B: A compact synthetic vaccinia virus early/late promoter for protein expression. Biotechniques 1997, in press.

21. Holzer G, Falkner FG: Construction of a vaccinia virus deficient in the essential DNA repair enzyme uracil DNA glycosylase by a complementing cell line. J Virol 1997, 71:4997-5002.
The authors stably transfected a cell line with the vaccinia virus uracil DNA glycosylase gene, encoding an enzyme required for viral DNA replication. The cells were then used to isolate and propagate a mutant vaccinia virus with a deleted uracil DNA glycosylase gene. In non-permissive cells, prolonged early gene expression was detected, suggesting the potential of the mutant virus as an expression vector.

22. Tsung K, Yim JH, Marti W, Buller RML, Norton JA: Gene expression and cytopathic effect of vaccinia virus inactivated by psoralen and long-wave UV light. J Virol 1995, 70:165-171.

23. Ward GA, Stover CK, Moss B, Fuerst TR: Stringent chemical and thermal regulation of recombinant gene expression by vaccinia virus vectors in mammalian cells. Proc Natl Acad Sci USA 1995, 92:6773-6777.

24. Ramsey-Ewing A, Moss B: Recombinant protein synthesis in CHO cells using a vaccinia virus/bacteriophage T7 hybrid expression system. J Biol Chem 1996, 271:16962-16966.

25. Wyatt LS, Moss B, Rozenblatt S: Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. Virology 1995, 210:202-205.

26. Sutter G, Ohlmann M, Erfle V: Non-replicating vaccinia vector efficiently expresses bacteriophage T7 RNA polymerase. FEBS Lett 1995, 371:9-12.

27. Hu L, Esposito JJ, Scott FW: Raccoon poxvirus feline panleukopenia virus VP2 recombinant protects cats against FPV challenge. Virology 1996, 218:248-252.

28. Kochneva GV, Urmanov IH, Ryabchikova EI, Streltsov VV, Serpinsky OI: Fine mechanisms of ectromelia virus thymidine kinase-negative mutants avirulence. Virus Res 1994, 34:49-61.

29. Romero CH, Barrett T, Chamberlain RW, Kitching RP, Fleming M, Black DN: Recombinant capripoxvirus expressing the hemagglutinin protein gene of rinderpest virus: protection of cattle against rinderpest and lumpy skin disease. Virology 1994, 204:425-429.

30. Jackson RJ, Hall DF, Kerr PJ: Construction of recombinant myxoma viruses expressing genes from different intergenic sites without associated attenuation. J Gen Virol 1996, 77:1569-1575.

31. Somogyi P, Frazier J, Skinner MA: Fowlpox virus host range restriction: gene expression, DNA replication, and morphogenesis in nonpermissive mammalian cells. Virology 1993, 197:439-444.

32. Britton P, Green P, Kottier S, Mawditt KL, Penzes Z, Cavanaugh D, Skinner MA: Expression of bacteriophage T7 RNA polymerase in avian and mammalian cells by a recombinant fowlpox virus. J Gen Virol 1996, 77:963-967.

33. Feng Y, Broder CC, Kennedy PE, Berger EA: HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane G protein-coupled receptor. Science 1996, 272:872-877.
Outstanding example of the use of the bacteriophage T7-vaccinia virus expression system for screening a cDNA library. This paper describes the discovery of an HIV-1 coreceptor molecule.

34. Hoffman MA, Banerjee AK: An infectious clone of human parainfluenza virus type 3. J Virol 1997, 71:4272-4277.

35. Bridgen A, Elliott RM: Rescue of a segmented negative-strand RNA virus entirely from cloned complementary DNAs. Proc Natl Acad Sci USA 1996, 93:15400-15404.
The first report of an infectious segmented RNA virus formed from cDNA. The bacteriophage T7-vaccinia virus expression system was used.

36. Petit DL, Koothan T, Liao D, Malinow R: Vaccinia virus transfection of hippocampal slices. Neuron 1995, 14:685-688.

37. Katz E, Wolffe EJ, Moss B: The cytoplasmic and transmembrane domains of the vaccinia virus B5R protein target a chimeric human immunodeficiency virus type 1 glycoprotein to the outer envelope of nascent vaccinia virions. J Virol 1997, 71:3178-3187.
The first report of the presentation of a recombinant protein on the surface of vaccinia virus particles. Further work along these lines may lead to targeting of virus particles to specific cells.

38. Brochier B, Costy F, Pastoret PP: Elimination of fox rabies from Belgium using a recombinant vaccinia-rabies vaccine: an update. Vet Microbiol 1995, 46:269-279.